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Protocols

Application of sequence-independent amplification (SIA) for the identification of RNA viruses in bioenergy crops

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A B S T R A C T

Miscanthus × *giganteus*, energycane, and *Panicum virgatum* (switchgrass) are three potential biomass crops being evaluated for commercial cellulosic ethanol production. Viral diseases are potentially significant threats to these crops. Therefore, identification of viruses infecting these bioenergy crops is important for quarantine purposes, virus resistance breeding, and production of virus-free planting materials. The application is described of sequence-independent amplification, for the identification of RNA viruses in bioenergy crops. The method involves virus partial purification from a small amount of infected leaf tissue (miniprep), extraction of viral RNA, amplification of randomly primed cDNAs, cloning, sequencing, and BLAST searches for sequence homology in the GenBank. This method has distinct advantage over other virus characterization techniques in that it does not require reagent specific to target viruses. Using this method, a possible new species was identified in the genus *Marafivirus* in switchgrass related to *Maize rayado fino virus*, its closest relative currently in GenBank. *Sugarcane mosaic virus* (SCMV), genus *Potyvirus*, was identified in *M.* × *giganteus*, energycane, corn (*Zea mays*), and switchgrass. Other viruses identified were: *Maize dwarf mosaic virus* (MDMV), genus *Potyvirus*, in johnsongrass (*Sorghum halepense*); *Soil borne wheat mosaic virus* (SBWMV), genus *Furovirus*, in wheat (*Triticum aestivum*); and *Bean pod mottle virus* (BPMV), genus *Comovirus*, in soybean (*Glycine max*). The method was as sensitive as conventional RT-PCR. This is the first report of a *Marafivirus* infecting switchgrass, and SCMV infecting both energycane and *M.* × *giganteus*.

1. Introduction

Bioenergy crops include crops used as feedstock for liquid biofuel production (FAO, 2008). They can be crops with high biomass yield for lignocellulosic ethanol production (biomass crops); crops that produce large amount of products like sugar, starch for ethanol production (sugar and starch crops); and crops from which commercial quantity of vegetable oil (biodiesel crops) can be extracted for biodiesel production. The abundance of biomass makes them attractive as renewed bioenergy feedstock, and more studies are being conducted to improve biomass crops.

Miscanthus × *giganteus*, *Saccharum* sp. (energycane), and switchgrass are three important bioenergy grasses with potential for lignocellulosic ethanol production because of their high biomass yields (Lewandowski et al., 2003; Samson et al., 2005). The high photosynthetic efficiency in the three species is related to their C4 photosynthetic pathway, leading to enhanced carbon sequestration (Sanderson and Adler, 2008; Heaton et al., 2004).

M. × *giganteus* is a natural hybrid of *Miscanthus sinensis* and *Miscanthus sacchariflorus* (Rayburn et al., 2009). It is a perennial crop native to Asia, and has a high biomass yield and an enormous energy potential because of its high cellulose content. It is non-invasive because it produces sterile seeds (Lewandowski et al., 2003). Rhizome cuttings are used to propagate *M.* × *giganteus*. At maturity, it stands approximately 3.5 m tall (Lewandowski et al., 2003) and dry matter yields above 30 ton ha^{-1} year^{-1} have been reported in Europe (Angelini et al., 2009; Lewandowski et al., 2000); and greater than 60 ton ha^{-1} year^{-1} has been reported also in the state of Illinois in the USA (Heaton et al., 2008).

Switchgrass is a perennial grass indigenous to Central and North America (Parrish and Fike, 2005). In areas with adequate rainfall, sustainable yields of about 15 ton ha^{-1} year^{-1} are achievable. However, more recently, peak dry mass yields from 26 to 38 ton ha^{-1} year^{-1} were reported in Illinois (Heaton et al., 2008). Switchgrass generally is shorter than *M.* × *giganteus*, but can grow up to 3 m high (Hultquist et al., 1996; Lemus et al., 2002). Unlike

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M. × *giganteus*, switchgrass is propagated mainly through seeds (Lewandowski et al., 2003; Sill, 1957).

Energycane is a hybrid of cultivated and wild sugarcane species (*Saccharum* sp.) that was selected for its high fiber content instead of high sugar (syrup) content for which commercial sugarcane is selected (Mislevy et al., 1995, 1993, 1992; Woodard et al., 1991). The purpose for this selection was to produce high energy content feedstock for lignocellusoic ethanol production. Viruses infecting existing commercial sugarcane have been characterized. It is expected that energycane varieties, being new inter-species crosses, may react differently to known pathogens of sugarcane. It is therefore important to evaluate released energycane varieties for reactions to known pathogens of sugarcane, and look out for new disease symptoms and identify their causal agents. To our knowledge, no viruses of energycane have been reported.

Since the biomass yield of bioenergy crops is critical, one desirable trait is resistance to diseases and pathogens that can cause reduction in biomass yield. Additionally, the crop should not harbor pathogens that can spread to nearby cultivated food crops, like cereals.

To our knowledge, virus infection of *M.* × *giganteus* in North America has not been reported. However, *Barley yellow dwarf virus* infection of *Miscanthus sacchariflorus* and *Miscanthus sinensis* 'Giganteus' (same as *M.* × *giganteus*) in the UK in plants grown from tissue culture imported from Germany (Christian et al., 1994) have been reported. In addition, the infection of *M. sacchariflorus* with *Miscanthus streak virus* has been reported also in Japan (Chatani et al., 1991). *Panicum mosaic virus* (Sill, 1957), *Sugarcane mosaic virus* and barley yellow dwarf viruses (Garrett and Dendy, 2004; Schrotenboer and Malmstrom, 2009) have been reported in switchgrass in the USA.

Because *M.* × *giganteus,* energycane and switchgrass could be cultivated in large scale for biomass purposes, it is important that pathogens infecting these crops and their impacts on biomass yield be identified and characterized to enable the development of control methods to prevent pathogen/disease spread. Potential methods for the control of diseases of these crops may include breeding for resistance and production of pathogen-free rhizomes (clean stock). Production of clean stock is vital for clonally propagated materials.

Identifying uncharacterized viruses infecting plants is challenging because there are no universal (for example, internal transcribed sequence (ITS)-like) primer sequences useful for polymerase chain reaction (PCR) detection like those available for many bacterial and fungal pathogens. Viruses lack common conserved regions within their genomes that can be used for this purpose. Currently, the most common methods for detecting simultaneously multiple viruses in plants include: multiplex polymerase chain reaction (m-PCR) (Agindotan et al., 2007; Wei et al., 2009), enzyme-linked-immunosorbent assay (ELISA) (Voller et al., 1976; Koenig, 1978), macroarray/microarray detecting methods (Engel et al., 2010; Agindotan and Perry, 2008), and double-stranded (ds) RNA isolation (Balijja et al., 2008; Susaimuthu et al., 2007). Each of these methods requires pathogen-specific reagents, like antibodies specific for a virus, or virus specific primers for sequence-dependent amplification. For detection and identification of unknown viruses, sequence-independent amplification is highly desired.

Different forms of sequence-independent amplification (SIA) methods have been used to identify new viruses in clinical samples (Ambrose and Clewley, 2006; Berthet et al., 2008; Djikeng et al., 2008). One of them is sequence-independent single primer amplification (SISPA) method; first described by Reyes and Kim (1991). This method has since been modified. The original SISPA involves ligation of both blunt ends of DNA molecules with asymmetric linkers (linker-adapter ligation), followed my amplification with a single primer with sequence complementary to the linker. Another sequence-independent amplification (SIA), a variant of SISPA, that eliminates the need for an adaptor ligation is one based on random PCR (rPCR) (Bohlander et al., 1992). It uses a first primer which has a 5′-end with distinct nucleotide sequence consisting of restriction and universal priming sites for subsequent cloning and amplification, and a 3′-end which consisting of a random hexamer or heptamer for priming with RNA or DNA. The subsequent amplification is done with a second primer complementary to the universal priming sequence at the 5′-end of the first primer. This variant has been used for the amplification of viral genomes (Agindotan and Perry, 2007, 2008; Froussard, 1992). Combining SIA with target enrichment, cloning, and sequencing have led to the identification of previously uncharacterized viruses in clinical samples (Allander et al., 2001; Victoria et al., 2008).

In some cases, sequence-independent amplification (SIA) has been used in macroarray/microarray to amplify total nucleic acids, followed by virus-specific oligonucleotide probe detection of plant viruses (Agindotan and Perry, 2007; Grover et al., 2010). SIA has been used also to amplify extracted dsRNA, followed by sequencing to identify plant viruses (Susaimuthu et al., 2007; Maccheroni et al., 2005). In later case, large amounts of plant samples were required and the targets were purified viral ds RNA.

In this report, the application of SIA is described for the identification of RNA viruses infecting bioenergy crops using RNA extracted from minipreps of infected leaf extracts.

2. Materials and methods

2.1. Samples

Two sets of symptomatic leaf samples: those that have been confirmed infected with specific viruses, and those that have not been tested for viruses. Others were healthy-appearing corn (*Zea mays*, cultivar unknown) and switchgrass (variety Cave-In-Rock) leaf tissues from plants in growth chambers (Table 1).

Those infected with known specific viruses were leaf samples of johnsongrass (*Sorghum halepense*), corn, soybean (cv. Williams 82), and wheat (*Triticum aestivum*, cultivar unkown). These plants were infected with *Maize dwarf mosaic virus* (MDMV), *Sugarcane mosaic virus* (SCMV), *Bean pod mottle virus* (BPMV), and *Soilborne wheat mosaic virus* (SBWMV), respectively (Table 1).

The infected johnsongrass and corn leaf tissues with mosaic symptoms were from plants maintained in the greenhouse and provided by Prof. Jerald K. Pataky, Department of Crop Science, University of Illinois, Urbana-Champaign, USA. The identities of the viruses in these two plants were kept secret from the investigators until these were identified. The infected wheat was from an experimental field in the University of Illinois, Urbana, while the BPMV-infected soybean was from a greenhouse at the University of Illinois, Urbana-Champaign.

Plants infected with unknown viruses were *M.* × *giganteus*, switchgrass and energycane. *M.* × *giganteus* leaves with yellow mosaic symptom (Fig. 1B) were from plants in experimental fields at Fairfield and Savoy, IL, and in a greenhouse at the University of Illinois, Urbana-Champaign (Table 1). Switchgrass leaf tissues with yellow mosaic symptoms (Fig. 1A) were collected from plants in experimental fields in Brownstown and Savoy, IL, and in Madison, WI. The switchgrass variety from Illinois was Cave-In-Rock, and the one from Wisconsin was a hybrid coded WI-SG-354B-Row2 (Table 1). Energycane (variety: L79-1002) stems were supplied by Dr. William Anderson, USDA/ARS Crop Genetics and Breeding Research Unit, Tifton, Georgia. The energycane stems were initially grown in a growth chamber and then moved to a greenhouse. While in the growth chamber, their leaves developed mosaic symptoms that were prominent on the main vein and slightly on leaf lami-

Table 1
Viruses identified using the sequence-independent amplification.

Leaf	Sampling date	Symptom	Source	[a]Identified viruses	[b]Viral insert	Genome coverage (%)	Mean contig length (nt)	Contig No.	[c]% ID	[d]GenBank Accession nos.
M. × *giganteus*-1	June 2009	Yellow mosaic	Fairfield, IL	SCMV	2/5	8	755	1	97	HM133586
M. × *giganteus*-2	June 2008	Yellow mosaic	Urbana (GH), IL	SCMV	2/3	16	768 ± 69	2	98	HM133585
M. × *giganteus*-3	August 2008	Yellow mosaic	Savoy, IL	SCMV	8/9	15	367 ± 149	3	98	GU068589
Switchgrass-1	June 2009	Mosaic	Brownstown, IL	MRFV	3/5	6	576	1	76	HM133582
Switchgrass-2	August 2009	Mosaic	Madison, WI	MRFV	3/5	8	731	1	80	HM133581
Switchgrass-3	August 2008	Yellow mosaic	Savoy, IL	MRFV	11/11.	23	730 ± 395	2	77	GU068591
Switchgrass-4	February 2010	Asymptomatic	Urbana (GC), IL	SCMV	2/5	6	265 ± 75	2	99	HM133587
Johnsongrass	July 2008	Mosaic	Urbana (GH), IL	MDMV	32/32.	34	645 ± 234	5	94	GU068590
Sweetcorn	July 2008	Mosaic	Urbana (GH), IL	SCMV	17/17.	33	617 ± 185	7	99	HM133588
Soybean W82	October 2008	leaf mottling	Urbana (GH), IL	BPMV RNA2	5/5.	43	1573	1	99	HM133580
Energy cane	July 2010	Yellow mosaic	Tifton, GA	SCMV	1/5	8	728	1	97	HM196762
				SBWMV RNA1	1/5	11	759	1	99	HM133583
Wheat	May 2008	Asymptomatic	Urbana, IL	SBWMV RNA2	2/5	64	788	1	99	HM133584

GC is the growth chamber; GH is the green house.
[a] SCMV: *Sugarcane mosaic virus*; MRFV: *Maize rayado fino virus*; SBWMV: *Soilborne wheat mosaic virus*; MDMV: *Maize dwarf mosaic virus*; BPMV: *Bean pod mottle virus*.
[b] Actual number of viral sequences divided by all the sequences obtained. Four sequences were bacterial; the rest viral, 19 host plant, and 89 viral.
[c] Percentage identity (ID) with respect to the most identical reference viral sequence in the GenBank. The reference viral sequence GenBank Accession numbers were: EU0191075 (SCMV), AF265566 (MRFV), AM110758 (MDMV), AF394607 (BPMV) and L07937 (SBWMV RNA 1) and AY016008 (SBWMV RNA 2).
[d] These were sequences we submitted to GenBank.

Fig. 1. Mosaic symptoms associated with identified viruses in: (A) Switchgrass; (B) *Miscanthus* × *giganteus* and (C) Energycane.

nas (Fig. 1C). Energycane leaf tissue with mosaic symptom in the growth chamber was tested for viruses.

Leaf tissues from fields were kept in a cooler containing ice for 1–3 days, between sampling and transportation. They were kept at 4 °C for 2–3 days, on arrival at Urbana, IL, and later frozen at −70 °C for a week to a month before they were processed. Energycane leaf tissues were kept also at 4 °C for 3 days prior to processing. See Table 1 for sampling dates.

2.2. Virus minpreps

One gram of leaf tissue from each sample was ground into powder in liquid nitrogen and then extracted with 20 ml citrate buffer (0.2 M sodium citrate, pH 6.5, containing 1% sodium sulfite, 2% polyvinyl pyrollidone (FW: 40,000)). Extracts were placed in 25 × 89 ml polycarbonate tubes (Seton, USA) and plant debris was removed by differential centrifugation at 109,000 × *g* for 10 min at 7 °C in a T-1250 rotor inside a Sorval WX ultra 80 ultracentrifuge (Thermo Scientific, Wilmington, MA, USA). Virus particles were precipitated by centrifugation at 109,000 × *g* for 1 h at 7 °C as above. The pellet was re-suspended in 500 μl of 50 mM sodium phosphate buffer (pH 7.0) and kept at 4 °C overnight. After thorough but gentle mixing with pipette, the suspension was clarified at 10,000 × *g* for 5 min at 4 °C. Aliquots of 200 μl of purified virus were stored at −70 °C prior to RNA extraction. See Fig. 2 for method flow chart.

2.3. Preparation of RNA from minipreps

RNA was extracted from 200 μl of each of the partially purified virus preparations (minipreps) in 900 μl RLT extraction buffer (with 2-mercaptoethanol) contained in RNeasy Plant Mini kit (Qiagen, Valencia,USA). Remnant DNA was digested with RNase-free, DNase 1 set (Qiagen) during the RNA purification, as recommended in the manufacturer's protocol. RNA was eluted with 30 μl of nuclease-free water. The amount of RNA was estimated with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, MA, USA).

2.4. RT-PCR

Viral RNA was amplified essentially as described by Agindotan and Perry (2007). Briefly, the reverse transcription reaction mixture consisted of 10 μl of DNA-digested viral RNA preparation and 2 μl of 10 μM random anchored primer (5′-TGGTAGCTCTTGATCANNNNNN-3′: Bohlander et al. 1992). The mixture was heated for 5 min at 65 °C and immediately chilled

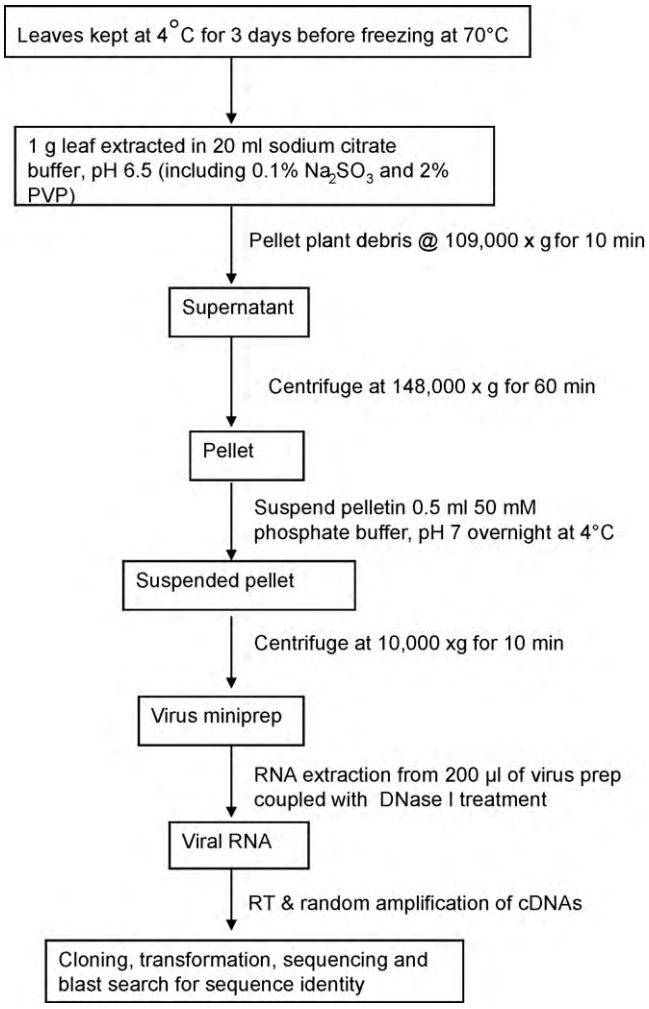

Fig. 2. The scheme of sequence-independent amplification coupled with target enrichment, cloning and sequencing.

on ice for 2 min. To the chilled mixture, 4 μl of 5× first-strand buffer (Fermentas, Glen Burnie, MD, USA), 2 μl 10 mM dNTP mix (Fermentas), 1 μl RiboLock™ RNase Inhibitor (40 U/μl; Fermentas), 1 μl RevertAid™ Reverse Transcriptase (200 U/μl; Fermentas) were added. The mix was incubated at 25 °C for 10 min and 37 °C for 1 h. At the end of the reaction, 1 μl of RNase H (Fermentas) was added and the mix incubated at 37 °C for 30 min and 65 °C for 15 min.

The 50 μl PCR reaction mixtures consisted of 2.5 μl of RT reaction product, 0.5 μl 10 μM of random anchor primer, 2 μl of 10 μM anchor primer (5′-AGAGTTGGTAGCTCTTGATC-3′: Bohlander et al. 1992), 25 μl 2× PCR mix (Fermentas), and 20 μl sterile, de-ionized water. Anchor-primed amplification was done in a 2720 Thermal cycler (Applied Biosystems, Forster City, CA, USA) with an initial 3 min denaturation step at 94 °C; followed by 5 cycles of 94 °C for 30 s, 37 °C for 15 s, 40 °C for 15 s, 45 °C for 15 s, 50 °C for 15 s, 55 °C for 15 s, 72 °C for 2 min, and 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min; and a final extension step at 72 °C for 10 min.

2.5. Agarose gel electrophoresis, purification, and quantification of PCR products

Amplification was first confirmed by analyzing 10 μl of each PCR products on 2% agarose gels in 0.5× Tris–boric acid electrophoresis buffer (Sambrook and Russell, 2001) and stained with ethidium bromide. Each of the remaining amplified PCR products (40 μl) was purified using QIAquick PCR kit (Qiagen). Purified DNA from each PCR product was eluted in 30 μl of elution buffer (EB), and concentration measured.

2.6. Ligation, transformation and cloning

Ligation mixture consisted of 2 μl (11–41 ng/μl) of purified fresh PCR product, 0.5 μl of 0.1 M salt solution, and 0.5 μl pCR®4.1-TOPO® vector (TOPO TA cloning® kit for sequencing: Invitrogen, Carlsbad, CA, USA). Ligation was done at room temperature for 30 min and One-Shot TOP®10 chemically competent *E. coli* (Invitrogen) was used for transformation, following the manufacturer's protocols, except that we used half the recommended competent cells were, to reduce cost. Two volumes of each transformed cell culture (20 and 50 μl) were plated in two LB-ampicillin plates and incubated overnight at 37 °C.

Single colonies (at least five) were picked and subcultured overnight in LB-ampicillin (100 μg/ml ampicillin) broth in a shaker at 250 rpm and 37 °C. Plasmid DNA was extracted from 1 ml of each pure culture using PureLink™ Quick Plasmid Miniprep kit (Invitrogen, USA) and eluted with 30 μl of TE buffer, following the manufacturer's protocol.

2.7. Sequencing and BLAST search

Purified plasmid DNA preparations were sequenced using M13 forward primer in an ABI 3730XL capillary automated sequencer (Applied Biosystems, USA) at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center, University of Illinois, Urbana-Champaign, USA. Sequence identity was determined using the Basic Local Alignment Search Tool (BLAST), available on the website of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Vector NTI Advance™11 software was used for global sequence alignment, contig assembly, percentage identity calculations. The fraction of viral (insert) sequences was calculated as the number of sequences of a specific virus identified divided by total number of sequences.

2.8. Comparing sensitivity of the generic method and RT-PCR, and ELISA

2.8.1. Leaf extract dilutions, RNA extraction for ELISA and RT-PCR

Leaf tissues of switchgrass infected with MRFV, corn infected with SCMV, and uninfected leaves of these plants, raised in a growth chamber, were used for these experiments. The infected leaf tissues were extracted (1 g in 20 ml of citrate extraction buffer) as described in section 2.2, and 10-fold serial dilutions made, to obtain infected extract dilutions of 1:20, 1:200, 1:2000, 1:20,000, and 1:200,000. The dilutions were made in uninfected leaf extract (1:20 dilution) of the corresponding plants. The diluted extracts were applied to microtiter plate at 100 μl/well.

From 20 ml of each diluted crude extracts (from 1 g of leaf tissue), 0.5 ml of miniprep was made as described in Section 2.2.

Viral RNA purified from 200 μl of each of the virus partial preparations was eluted in 30 μl of elution buffer, as described in Section 2.3. Ten microliters of the RNA was used for 20 μl reverse transcription reactions, for both virus-specific RT-PCR and random RT-PCR.

2.8.2. DAS-ELISA

The 10-fold serial dilutions of crude leaf extracts of SCMV-infected corn and uninfected (healthy) corn of 1:20 dilution, and the corresponding minipreps made from the diluted extracts, were tested for SCMV using Agdia testing kit (Agdia Inc., USA), and following the manufacturer's ELISA protocol. The crude extracts were applied at 100 μl/well. Minipreps were diluted 40-fold to bring them to original dilution of the infected crude extracts from which

they were prepared, and applied at 100 μl/well. That is, both the leaf extract and miniprep starting dilution was equivalent to 50 mg (0.5 mg/μl) of virus-infected leaf tissue

2.8.3. Comparing the sensitivities of sequence-independent amplification (SIA) method and the conventional RT-PCR

The sensitivities of the sequence-independent amplification (SIA) and virus-specific conventional RT-PCR were determined using purified RNAs from minipreps obtained from 10-fold serial dilutions of extracts of leaves infected with SCMV and MRFV (as described in Section 2.8.1). For the SIA method, the protocols in Sections 2.4–2.7 were followed. The conventional RT-PCR method was identical to the random RT-PCR procedure of the SIA method except that virus-specific primers were utilized. Reverse transcription was at 42 °C, and PCR cycling conditions were: 94 °C for 3 min and 40 cycles of 94 °C for 30 s, 50 °C (for SCMV) or 60 °C (for MRFV) for 30 s; and 72 °C for 30 s (SCMV) or 45 s (MRFV), and final extension at 72 °C for 10 min and a hold at 4 °C.

The primers used for the detection of the MRFV from switchgrass were B088-MRFV-10R: GCCCACAGGTCTTATGGCCGACCTGCTACC and B089-MRFV-F-switchgrass: GCTATTCCTGCTCCTCCTCGTGTG-GTTGAAACC). B089-MRFV-F-switchgrass was a modification of the MRFV-10F primer reported for the detection of MRFV from *Z. mays* (Hammond et al., 1997). The modification was based on the sequence (GenBank Accession no. GU068590) of the MRFV isolate we obtained from switchgrass and submitted to the GenBank. The expected amplicon size was 635 bp.

Likewise, the primers used for the detection of SCMV (B104-SCMV FP: ATTTCTTCGTCGCCATACCGGAGA and B106-SCMV RP: AAGTGTGGACACGGACCTTTGACA) were designed from the sequence (GenBank Accession no. GU068589) of the virus isolate we obtained from *M.* × *giganteus* and submitted to the GenBank. The expected amplicon size was 210 bp.

2.9. Effects of differential centrifugation on identification of viruses from minipreps

Leaf extracts (1:20 dilution in extraction buffer) of switchgrass infected with MRFV, corn infected with SCMV, and leaf extracts of the uninfected plants (described in Section 2.6.1) were each centrifuged at three different centrifugal forces to remove host plant materials from the minipreps. This was to determine the optimum force for this step of the miniprep procedure (Fig. 2). The forces were: 76,000 × *g*, 109,000 × *g*, and 148,000 × *g*, at 7 °C for 10 min. Other steps of the sequence-independent amplification method were followed, to identify the viruses in each of the 12 samples (see Sections 2.2–2.7). Five transformed bacterial colonies were picked from each of the 12 transformations, and sequenced.

3. Results

3.1. Identification of viruses by sequence-independent amplification (SIA)

We applied the SIA method (Fig. 2) and identified viruses from infected leaf samples (Table 1).

Sugarcane mosaic virus (SCMV), genus *Potyvirus*, was identified in *M.* × *giganteus*, switchgrass, energycane, and corn leaf samples. A virus, most closely related to *Maize rayado fino virus* (MRFV), genus *Marafivirus*, was identified in switchgrass from fields in Illinois and Wisconsin. This virus for now is referred to as MRFV isolate from switchgrass. Other viruses identified with the SIA method included *Soil-borne wheat mosaic virus* (SBWMV), genus *Furovirus,* in wheat; *Maize dwarf mosaic virus* (MDMV), genus *Potyvirus,* in johnsongrass; and *Bean pod mottle virus* (BPMV), genus *Comovirus*, in soybean.

The nucleotide sequences of viral clones from each of the identified viruses were aligned and contigs with or without gaps were submitted to the GenBank and their Accession numbers shown in Table 1.

3.2. Effect of triton x-100 on minipreps related to virus identification

Triton x-100 is a detergent used in virus purification to remove the host plant green pigment (chlorophyll), liberate viruses from membranes, and increase virus yield. We examined the effect of triton x-100 on virus detection using SIA method. Corn and switchgrass-3 leaf tissues infected with SCMV and MRFV (Table 1) respectively, were partially purified from 1:20 diluted extracts with and without triton x-100. The detergent was added at 0.5% to supernatant obtained after the first differential centrifugation (Fig. 2). The supernatant was mixed for 30 min at 4 °C. Other steps of the SIA method were followed to identify viruses in these samples.

The results showed that SCMV and MRFV were identified with equal efficiencies in the corn and switchgrass minipreps with or without triton x-100 treatment. All the sequences (5/5) identified in each test were viral. Consequently, in subsequent tests, leaf extracts were not treated with triton x-100.

3.3. RNA yield and virus detection

We examined the yields of RNA extracted from 200 μl each of minipreps of virus-infected and uninfected leaf tissues for correlation with virus identification.

From Fig. 6D, the RNA yields obtained from minipreps of some infected leaf extracts (1 in 20 extract dilutions) were higher than those from the corresponding uninfected samples. This was true for the SCMV-infected corn and MRFV-infected switchgrass-3 (Table 1). However, as these virus-infected extracts were diluted further with uninfected extract, these differences decreased and then ceased. In infected leaf samples at 1:2000 and 1:20,000 dilutions, the amounts of recovered RNA were below the detection limit of Nanodrop spectrophotometer.

The mean amounts of RNA extracted from 200 μl of miniprep (prepared from 400 mg of leaf tissue) with and without DNase 1 treatment were 69 ± 70 and 1386 ± 1013 ng (n = 100), respectively, indicating that large amount of nucleic acids were removed after DNase treatment.

3.4. Random amplification, cloning and sequencing

Randomly amplified PCR products appeared as smears in 2% agarose gels (Fig. 4 is representative of rRT-PCR products). Most of the PCR products were 0.5 kb and above. Viruses were identified also in samples with no detectable RNA. The viral genome coverage [(sum of sizes of viral contigs/size of virus genome) × 100%] ranged from 6% to 64% (Table 1 and Fig. 5). From Table 1, 80% of the sequences obtained were viral, 16% were host plant, and 4% bacterial. The sequences of non-viral clones were not submitted to the GenBank. The mean contig length obtained from the viral clones ranged from 265 to 1573 nt (Table 1).

3.5. Effects of ultracentrifugation forces on virus identification

Removal of host plant materials while minimizing virion losses was vital for efficient identification of viruses by the SIA method. A step in the miniprep procedure was a differential centrifugation to remove host plant materials (membranes, organelles and leaf tissues). Three forces: 76,000 × *g*, 109,000 × *g* and 148,000 × *g* were applied on leaf extracts (1:20 dilution) of SCMV-infected

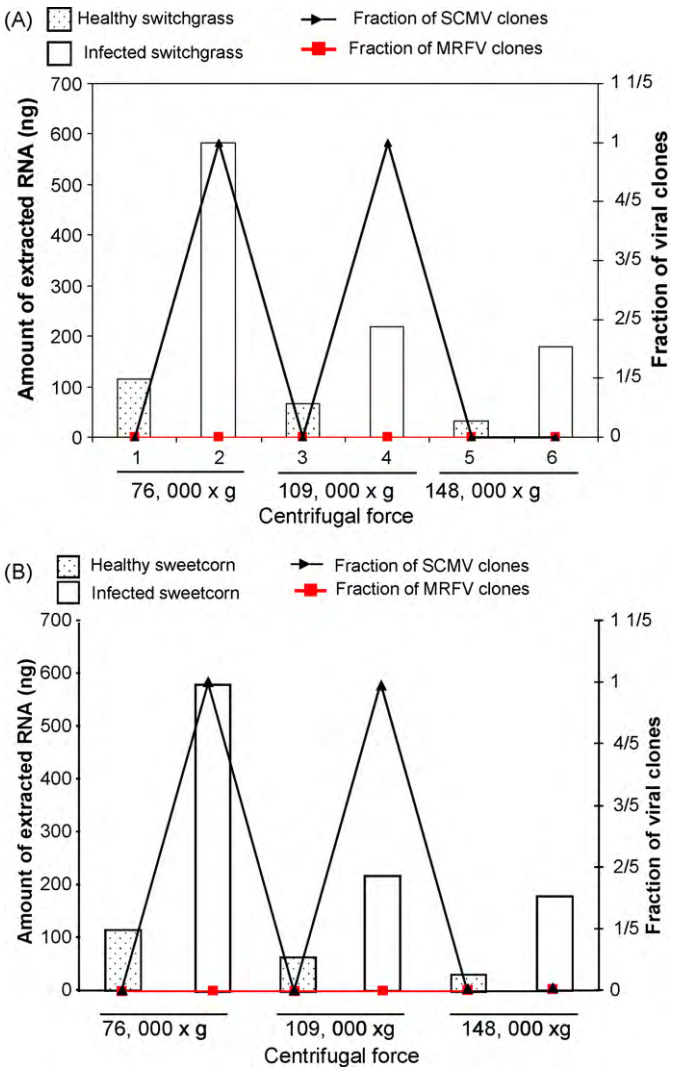

Fig. 3. Effects of differential centrifugal forces on amount of RNA extracted from minipreps and on the fraction of viral sequences identified. (A) Leaf sample: "Healthy" and "Infected" switchgrass (Cave-In-Rock). The bar chart: The amount of extracted RNA versus centrifugal force; The line graph: fraction of viral clones versus centrifugal force. (B) Leaf sample: "Healthy" and "Infected" sweet corn. The bar chart: the amount of extracted RNA versus centrifugal force; the line graph: fraction of viral clones versus centrifugal force. RNA was extracted from 200 μl of miniprep (equivalent to 0.4 mg of leaf tissue).

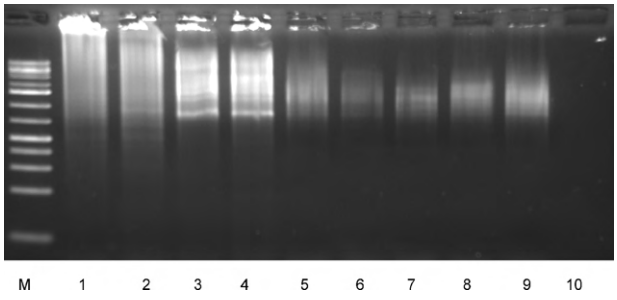

Fig. 4. Randomly amplified cDNA from RNA extracted from leaf minipreps. M: 1 kb plus DNA marker (Fermentas), 1: MRFV-infected switchgras, 76,000 × *g*; 2: MRFV-infected switchgrass, 109,000 × *g*; 3: SCMV-infected corn, 76,000 × *g*; 4: SCMV-infected corn, 109,000 × *g*, 5: "Healthy" switchgrass, 76,000 × *g*; 6: "Healthy" switchgrass, 109,000 × *g*; 7: "Healthy switchgrass, 148,000 × *g*; 8: "Healthy" corn 109,000 × *g*; 9: "Healthy sweetcorn, 76,000 × *g*; 10: water control. The centrifugal forces were used to remove host plant materials during partial purification of leaf extracts.

corn, "healthy" corn, MRFV-infected switchgrass-3 and "healthy" switchgrass-4 (Table 1 and Fig. 3), while keeping constant other factors. The highest amount of RNA was recovered at 76,000 × *g*. With any of the centrifugal forces, viruses were detected in the infected samples. SCMV was identified in healthy-looking switchgrass only when the centrifugal force of 76,000 × *g* was applied. The virus was not detected when the other centrifugal forces were applied. In addition, the fraction of viral inserts increased for MRFV-infected samples as the centrifugal force was decreased from 148,000 × *g* to 76,000 × *g*.

3.6. Sensitivity of the SIA method

The SIA method sensitivity was compared with virus-specific RT-PCR for the identification of SCMV in corn and MRFV in switchgrass-3 (Table 1 and Fig. 6). The two methods: SIA (Fig. 6A) and virus-specific RT-PCR (Fig. 6B), detected the two viruses in leaf extract up to 1:20,000 dilution. The MRFV and SCMV primers amplified the expected size of amplicons (635 bp for MRFV, and 210 bp for SCMV).

SCMV was detected by DAS-ELISA in leaf extracts and corresponding minipreps up to 1 in 2000 dilution (Fig. 3c), indicating both had similar amounts of virus.

3.7. Molecular characterization of identified viruses

Based on nucleotide sequences, the SCMV identified in *M.* × *giganteus*, switchgrass and corn were 97–99% identical to the corresponding sequences of a "SCMV-VER1" isolate from Mexico (EU 091075), while those of the MRFV identified in switchgrass were 78–80% identical to those of a "Costa Rican" isolate from corn (AF265566) (Table 1). Also, the nucleotide sequence of MDMV identified in johnsongrass was 94% identical to that of a "Sp" isolate from Spain (AM110758), while the identified BPMV nucleotide sequence from soybean was 99% identical to a "K-Hancock 1" strain from USA. The nucleotide sequence of SBWMV RNA-1 identified in wheat was 99% identical to a "US-Nebraska, 1981 wild-type" isolate (L07937), while the SBWMV RNA-2 nucleotide sequence identified from the same sample was only 88% identical to the RNA-2 (L07938) of the same isolate, but 99% identical to the "New York" strain from USA (AY016008).

The predicted partial polyprotein with gaps (490 a.a.), of an isolate of MRFV identified in switchgrass from Savoy, was 81.4% identical to the corresponding polyprotein sequence (NP115454) of a "Costa Rican" isolate of MRFV from *Z. mays*. A sequence of the polyprotein (225 a.a.) was 79% identical to an NTPase/helicase (NP_734075), and another 122 aa sequence was 72% identical to the capsid protein of MRFV (NP_734077) of the Costa Rican isolate. Because only the Costa Rican isolate of MRFV had its full capsid protein (CP) amino acid sequence available in the GenBank, we were able to compare a larger region of the CP. However, for other MRFV isolates, only their partial CP amino acid sequences were available, and as such a smaller region of their CPs was compared in Table 2.

From Table 2, the percentage identities of the common region of the available partial CPs of all MRFV isolates (61 a.a) relative to that obtained from switchgrass ranged from 75% to 80%. The MRFV isolate from USA (in *Z. mays*) was the least related (75% identity) and the Brazilian isolate (80% identity) was the most related to the isolate of switchgrass from Savoy, USA.

4. Discussion

A miniprep method was designed to remove host plant materials with minimum loss of virus particles, and to concentrate them. The observation that the virus titers of SCMV in infected leaf and

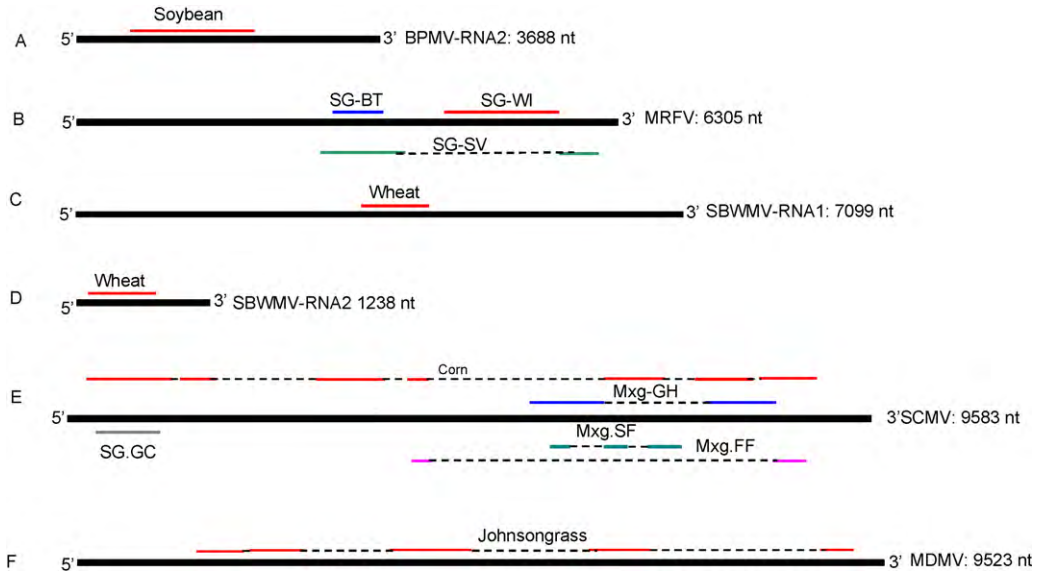

Fig. 5. Genome coverage of viral sequences identified using SIA-based method. (A) The bold black line represents referenced *Bean pod mottle virus* (BPMV) genome (GenBank Accession no.: AF394607). The red line represents the BPMV viral sequence we identified in soybean. (B) The bold black line represents referenced *Maize rayado fino virus* (MRFV) genome (GenBank Accession no.: AF265566). The blue, red and green lines represent the genome coverage of MRFV viral sequences we identified in switchgrass-1 (Brownstown, IL), switchgrass-2 (Madison, Wisconsin), and switchgrass-3 (Savoy, Illinois), respectively. (C) The bold black line represents referenced *Soilborne wheat mosaic virus* (SBWMV) RNA 1 genome (GenBank Accession no.: L07937). The red line represents the SBWMV RNA 1 sequence we identified in wheat. (D) The bold black line represents referenced *Soilborne wheat mosaic virus* (SBWMV) RNA 1 genome (GenBank Accession no.: AY016008). The red line represents the SBWMV RNA 2 sequence we identified in wheat. (E) The bold black line represents *Sugarcane mosaic virus* (SCMV) referenced genome (GenBank Accession no.: EU0191075). The gray, red, blue, green and pink lines represent SCMV sequences we identified in switchgrass, corn, *Miscanthus* × *giganteus* from the greenhouse, *M.* × *giganteus* from SoyFace, IL, *M.* × *giganteus* from Fairfield, IL, respectively. F: The bold black line represents referenced *Maize dwarf mosaic virus* (MDMV) genome (GenBank Accession no.: AM110758). The red line represents the MDMV viral sequence we identified in johnsongrass. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

the corresponding leaf miniprep were identical, suggested minimal virus loss during partial purification. Partial purification from little amount of leaf tissues have been in use to concentrate viruses for transmission electron microscopic studies and in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for virus coat protein characterization (Lin et al., 1977).

In the described partial purification, citrate buffer, pH 6.5, was used. Generally, buffer with pH close to neutral have been used to extract unknown viruses (Hull, 2009) and this seems to have worked for the SIA-based method. However, there are some viruses that are unstable at this pH, like *Tobacco necrosis virus* (TNV) (Finlay and Teakle, 1969) and *African cassava mosaic virus* (Kittelmann and Jeske, 2008); so, buffer of different pH will be suitable for such viruses. The stability of the capsid protein is vital for the protection of viral nucleic acid from degradation by host plant nucleases during the virus partial purification procedure (Alzhanova et al., 2001; King et al., 2001).

It is well known that ribosomes co-precipitate with viruses because they have similar sedimentation coefficient. So, there is the need to remove them from virus preparations (www.cipotato.org/csd/materials/pvtechs/Fasc5.1(99).pdf) because they may protect ribosomal RNA from degradation by host plant nucleases, and they may end up co-purified with viral RNA, as we have observed using the SIA-based method. We know that young and fresh leaf tissues are actively growing and are expected to produce a lot of ribosomes and RNA species (Thomas and Stoddart, 1980). Keeping detached leaf tissues in the cold room for a couple of days might have helped to reduce the amount of ribosomal RNAs in our samples because of the catabolic processes associated with leaf senescence (Srivastava and Arglebe, 1967).

Any detection method must be reliable and sensitive. The SIA-based method described was not only as sensitive as the conventional virus-specific RT-PCR method for detection of viruses; it in addition, identified the viruses to subspecies level. The sensitivity

Table 2
Comparison of partial capsid protein amino acid sequences (61 a.a) of MRFV isolates from *Panicum virgatum* (switchgrass) and *Zea Mays* (corn).

MRFV isolates[a]	MEX	**US-SG**	PE-B	US-MZ	VEN	GUA	ECU-A	COR-C	COL-A	BRA-17	BO-A
MEX		79	95	97	100	100	93	98	95	97	97
US-SG			77	75	79	79	78	77	79	80	79
PE-B				92	95	95	92	93	93	93	95
US-MZ					97	97	90	95	92	93	93
VEN						100	93	98	95	97	97
GUA							93	98	95	97	97
ECU-A								92	92	95	93
COR-C									93	95	95
COL-A										93	95
BRA-17											95
BO-A											

Apart from MRFV from switchgrass (US-SG), the rest were isolates from corn.

[a] MEX (Mexico: U97725.1), US-SG (switchgrass, USA), PE-B (Peru-B: U97727.1), US-MZ (*Zea mays*, USA: U97729.1), VEN (Venezuela: U97730.1), GUA (Guatemala: U97724.1), ECU-A (Ecuador, A: DQ196348.1), COR-C (Costa Rica, C: U97723.1), COL-A (Colombia, A: U97719.1), BRA-17 (Brazil, 17: AF186177.1), and BO-A (Bolivia, A: U97717.1). Where there were more than one isolate sequence of *Z. mays* per country, only the most related one to the switchgrass was included in the table.

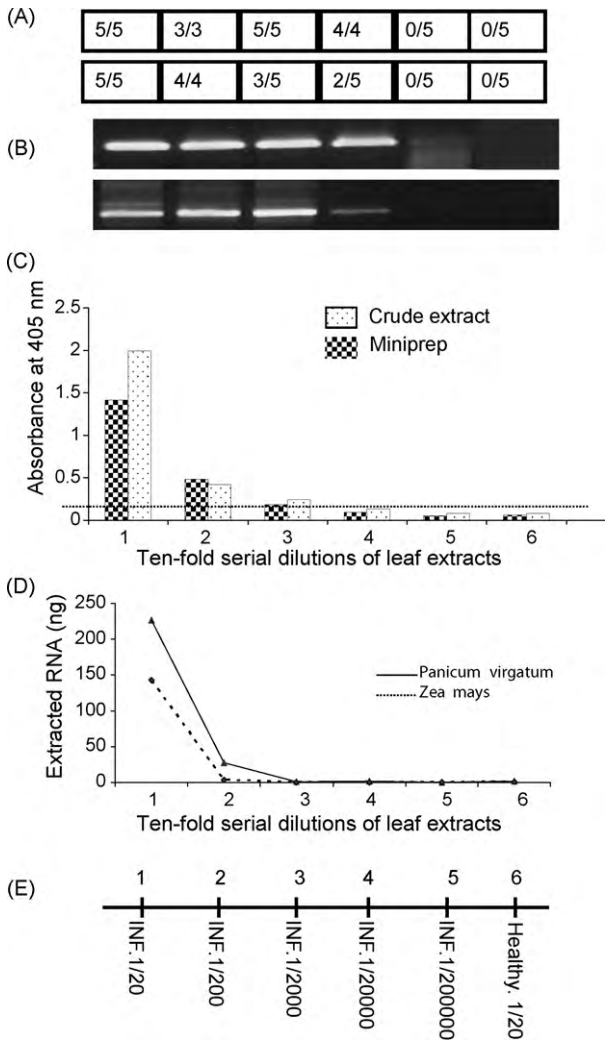

Fig. 6. Comparing the sensitivity of the sequence-independent amplification (SIA) method, specific RT-PCR and DAS-ELISA for the detection of SCMV in *Zea mays* and MRFV in *Panicum virgatum*. (A) SIA method for the detection of SCMV in Z. *mays* (first row) and detection of MRFV in *P. virgatum* (second row), in RNA extracted from minipreps of 10-fold serial dilution of leaf extracts. Each box in each row represents each dilution and the figure in it represents the fraction of viral sequences identified. (B) Virus-specific RT-PCR detection of SCMV in *Z. mays* (first row) and MRFV in *P. virgatum* (second row), from same set of RNAs as in "B". (C) DAS-ELISA for the detection of SCMV in *Z. mays* from 10-fold serial dilution of crude leaf extracts, and equivalent amount of minpreps from same extracts. The horizontal dotted line indicates the threshold absorbance value above which tested leaf extract dilution was considered positive for SCMV. The threshold value was twice the mean absorbance value of the "healthy" leaf sap. (D) Amount of extracted RNA from 200 ul miniprep. (E) A dilution line indicator. The numbers on top of the line correspond to the leaf extract dilution shown below the line, and these in turn correspond to the 10-fold serial dilution of the original crude leaf extracts from which minipreps and RNA were extracted, as described in figures "A–D". The amount of leaf extract, miniprep and RNA per test was equivalent to 3.33 mg of infected leaf tissue. Serial dilution of infected leaf extract was done in 1/20 diluted "Healthy" leaf extract.

of this method could be linked to the miniprep and RNA extraction procedures, and the random amplification at multiple sites (multi-locus priming) of viral genomes (Bohlander et al., 1992).

Our identification of MDMV in johnsongrass was consistent with the virus inoculated on the plant (MDMV), as later revealed by the sample provider. Johnsongrass, a weed, was used as a blind, positive control for the development of the SIA-based identification method. Identification of MDMV in johnsongrass in our preliminary investigation prompted extension of the method for the characterization of the viruses infecting *M.* × *giganteus,* energycane and switchgrass. SCMV was identified in corn, the second, blind, positive control. This was also in agreement with the virus the leaf tissue provider inoculated on the plant.

The identification of SCMV by this method is significant because, to our knowledge, this is the first report of SCMV infecting *M.* × *giganteus* and energycane. SCMV is distributed world-wide. It has been reported that at harvest, sugarcane stalks from SCMV-infected plots recorded a significant reduction in cane diameter (16%), cane weight (27%) and number of internodes (27%) (Viswanathan and Balamuralikrishnan, 2005; Singh et al., 2003). Based on the documentation of sugarcane yield loss, this virus could also potentially reduce biomass yield of *M.* × *giganteus* and energycane.

Sugarcane mosaic virus (SCMV) identified in *M.* × *giganteus* was identical to the ones detected in switchgrass, energycane and corn. This was not surprising as all the plants were from Illinois, except for energycane, and the virus is aphid transmitted. It seems this strain of SCMV is wide spread, as the identified SCMV isolates were highly identical to a Mexican isolate.

The virus identified in switchgrass is most related to *Maize rayado fino virus*, a type member of the genus *Marafivirus*. This is the first report of a *Marafivirus* infecting switchgrass in the field. All MRFV isolates with sequence accessions in GenBank were obtained from *Z. mays* (Hammond et al., 1997). Based on the partial capsid protein (CP) amino acid sequences, the MRFV isolates of switchgrass and *Z. mays*, both from USA, were more distantly related than the isolates of *Z. mays* from South America, suggesting that the MRFV isolate infecting switchgrass and *Z. mays* in America (USA) are different. In addition, the percentage identities of the nucleotide and amino acid sequences of the partial coat protein of the MRFV from switchgrass suggest that the virus may be a different species of the genus *Marafivirus* rather than a strain of the virus. This is because the *International Committee on Taxonomy of Viruses* (ICTV) criteria for delineation of species in the genus *Marafivirus* include overall sequence identity of less than 80% and CP sequences of less than 90% identity (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_tymov.htm#Genus2). To confirm this, a complete genome sequence of this virus needs to be determined, and its transmission and symptomatology need to be studied. For now, this virus is regarded as a switchgrass isolate of MRFV.

The infection of switchgrass by a *Marafivirus* is very important, as members of this genus have been known to cause severe yield losses. MRFV, a type member of this genus affects the photosynthetic system, causing leaf chlorosis and die-back (Gamez, 1973; Wolanski and Maramorosch, 1979). This may have significant effects on yield on C4 biosynthetic plants like switchgrass. This virus is transmitted by leafhoppers (Nault et al., 1980) and has been determined to cause severe yield losses (40–50%) in indigenous genotypes of *Z. mays* in Latin America and may cause 100% loss in some new cultivars (Bustamante et al., 1998). It is important to study the impacts of MRFV (Switchgrass isolate) infection on biomass yield of switchgrass; its transmission into *Z. mays,* and other cultivated cereals. In addition, there is need to determine if this virus can be transmitted to *M.* × *giganteus*, and energycane, as both plants could potentially be planted close to each other in production settings in the future.

Identification of the two RNA-1 and RNA-2 genomes of *Soil borne wheat mosaic virus* (SBWMV) from wheat is significant because only five clones were sequenced to obtain this information. The biapartite viral genomes seem to have come from two different SBWMV strains probably due to reassortment of genomes as a result of dual infection of two strains of the virus (Miyanishi et al., 2002). This is significant because this method enables identification of mixed infection of strains of SBWMV.

Bean pod mottle virus (BPMV) causes mottling and distortion of soybean seeds (Ziems et al., 2007). The significance of the detec-

tion of the virus from this plant is that it shows that the SIA-based method could be adequate for the identification of viruses from dicotyledonous plants.

SIA, as well other molecular identifications methods, only identifies pathogens and do not establish the etiological agent of a disease. Koch's hypothesis needs to be tested (Ambrose and Clewley, 2006).

We reported that viruses infecting *M.* × *giganteus* (SCMV), switchgrass (SCMV and MRFV) and energycane (SCMV) were associated with mosaic symptoms (dispersed leaf chlorosis) on the leaves of these plants. As shown in tobacco infected with *Tobacco mosaic virus* (TMV), the virus interferes with chloroplast development and function. The infection was associated with reduced size and number of chloroplasts, low chlorophyll content and low efficiency of CO_2 fixation in the chloroplast (Wilhelmova et al., 2005; Jensen, 1968). The impairment to the function of the chloroplast has been associated with the presence of the virus coat protein on this organelle (Reinero and Beachy, 1989; Hodgson et al., 1989). This is significant because being C4 plants, their choice as bioenergy crops is related to their superior photosynthetic abilities (Christin et al., 2009; Wang et al., 2007). Therefore, any stress that reduces these abilities will decrease their biomass yields; and it will do so yearly as the virus concentration increases in these perennial crops that are propagated mainly through stems and rhizome cuttings. Therefore, production of clean planting materials is an important management strategy for the control of the impact of the mosaic diseases cause by these viruses (Balamuralikrishnan et al., 2003).

For non-sterile seed-producing plants like switchgrass and energycane, screening for sources of resistance and breeding for resistant varieties against these viruses and other important viruses of these plants should be a research focus of high priority. Energycane and *M.* × *giganteus* are propagated through rhizome cuttings, so periodic planting of new clean rhizomes will help reduce disease incidence and impacts on the bioenergy crops and nearby cultivated food crops.

5. Conclusion

Appying the SIA-based method, RNA viruses were identified in *M.* × *giganteus*, switchgrass, energycane, johnsongrass, sweet corn, soybean and wheat. The five viruses identified were from four genera (*Potyvirus, Furovirus, Cumovirus* and *Marafivirus*), three families (*Potyvirida, Comoviridae, Tymoviridae),* and one virus unassigned to a family. These viruses were detected in five monocots and one dicotyledonous plants.

The method is recommended for the identification of uncharacterized RNA viruses, and it can be modified for the identification of DNA viruses. Once a virus has been identified with this method, primers can be designed based on the conserved region of the virus and used for routine amplification and detection of the virus.

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